

THE INTERMEDIARY METABOLISM OF PHENYLALANINE

BY
JOSEPH P. CHANDLER

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIRE-
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COMPARATIVE STUDIES OF THE METABOLISM OF THE AMINO ACIDS

V. THE OXIDATION OF PHENYLALANINE AND PHENYLPYRUVIC ACID IN THE ORGANISM OF THE RABBIT*

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The problems of the intermediary metabolism of the aromatic amino acids of the protein molecule, tyrosine, and phenylalanine, have been investigated by a number of experimental methods, of which perfusion of the acids and their possible intermediary catabolites through surviving organs and studies of their fate in the human organism in alkaptonuria or in the organisms of normal laboratory animals have been most productive. Excellent critical evaluation of this work is available in the summaries of Dakin (1), in 1922, and of Mitchell and Hamilton (2), in 1929. The data obtained have seldom been of a quantitative nature since methods for the exact determination of the various substances, whose existence as intermediary catabolites has been proved, have not been available. The results have had a qualitative significance, therefore, rather than a quantitative one, since the methods for the most part have involved isolation, a procedure inadequate from the quantitative view-point. In the present investigation an attempt has been made to study the excretion of some of the intermediary metabolites quantitatively and to determine the extent to which those reactions, which result in the formation of phenyl derivatives not readily oxidized further, occur after the feeding or injection of phenylethylamine, phenylalanine, and phenylpyruvic acid to rabbits.

* A preliminary report of part of this work was presented before the Twenty-fourth annual meeting of the American Society of Biological Chemists at Chicago (*J. Biol. Chem.*, **87**, p. lvi (1930)).

EXPERIMENTAL

Rabbits were given phenylalanine and related compounds orally or subcutaneously and the urine excreted during the period immediately following was examined for possible intermediate products, chiefly phenylpyruvic, phenylacetic, and benzoic acids, and the conjugation products of the last two with glycine.

Male rabbits were maintained on constant weighed diets of cabbage and oats over the period of study which in some cases exceeded 3 months in duration. Since the volume of urine excreted by the rabbit is so small that it is difficult to make a series of analyses which require considerable amounts of urine on 24 hour specimens, 48 hour samples were obtained and analyzed. Hydrolysis of hippuric or phenaceturic acid prior to the analysis was prevented by the addition of dilute nitric acid. Determinations of creatinine were made in order to secure a check on the completeness of the collection of the 48 hour samples.

Equal amounts of the substances under investigation were administered on 2 consecutive days, usually through a stomach tube. Two 2 gm. portions of phenylalanine or equivalent amounts of sodium phenylpyruvate were given during the experimental periods. Partial neutralization of the amino acid was necessary in order to dissolve it in a volume convenient for administration (usually 40 cc.). In a few experiments subcutaneous injection of the compound to be studied was employed in order to exclude possible chemical changes due to the activities of the intestinal microorganisms.

The phenylpyruvic acid, synthesized in this laboratory by the method of Hemmerle (3), because of the instability of the free acid was purified as the sodium salt and fed in this form. Since the sodium salt of the acid contains 1 molecule of water of crystallization, 1.25 gm. of the salt are equivalent to 1 gm. of the free acid and also to 1 gm. of phenylalanine. Very dilute solutions of the acid or salt gave the characteristic green color with ferric chloride. All of the preparations were examined for the presence of phenylacetic acid with negative results.

Methods of Analysis

The Wayne modification (4) of the Steenbock sublimation method for benzoic and phenylacetic acids was used in all the

quantitative experiments. A ground glass joint between the U-tube and the sublimation tube was found to be an improvement over the rubber stopper connection used by Wayne, since removal of the sublimate from the horizontal part of the U-tube by heating is difficult without burning the rubber stopper of the Wayne apparatus. Difficulty was experienced in applying this method to the determination of small quantities of benzoic acid and phenylacetic acid in mixtures. In experiments with known amounts of pure substances, the total weight of sublimate checked fairly well with the total amounts of acids taken, but the calculated proportions of benzoic and phenylacetic acids were frequently inconsistent. Although the method has been useful as indicating the approximate values, we have found it desirable to supplement the Wayne method with other determinations.

Phenylpyruvic acid is converted to phenylacetic acid under the conditions of the Wayne method and the phenylacetic acid derived from it appears in the sublimate. In experiments with different preparations of sodium phenylpyruvate, about 75 per cent of the theoretical amount of phenylacetic acid was obtained in the sublimate. It was at first believed that the observed formation of phenylacetic acid was due to the presence of an oxidizing agent (hydrogen peroxide) during the hydrolysis. Experiments in which the hydrogen peroxide was omitted, however, demonstrated that this was not the case. In one experiment, for example, 0.250 gm. samples of sodium phenylpyruvate (equivalent to 0.165 gm. of phenylacetic acid) were subjected to the procedure of the Wayne method both with and without the presence of hydrogen peroxide during hydrolysis. By the original method, 0.117 and 0.119 gm. of sublimate were obtained in duplicate determinations, while without the presence of the peroxide, the weights were 0.052 and 0.050 gm. The sublimed material in these experiments was identified as phenylacetic acid by recrystallization and determination of the melting point. From these and other similar experiments, we may conclude that the conversion of phenylpyruvic acid to phenylacetic is not complete by this method even when the peroxide is present and that some formation of phenylacetic acid occurs even in the absence of an oxidizing agent. Phenylalanine gave no sublimate in the Wayne method.

The behavior of phenyllactic acid¹ was also studied. Samples of 0.3 gm. were subjected to the procedure of the Wayne method both with and without the addition of hydrogen peroxide. In the presence of the oxidizing agent, the sublimed acid obtained amounted to 33.7 and 26.4 per cent of the amount of phenylacetic acid which could be derived by oxidation, while in the absence of peroxide, the amount of sublimate was almost negligible, 3.4 and 3.1 per cent of the value expected if complete conversion to the acetic acid derivative had occurred. It is evident that the amount of sublimable material obtained in the Wayne procedure from phenyllactic acid is much less than that obtained from phenylpyruvic acid especially in the absence of peroxide.

The method described by Griffith (5) for the determination of hippuric acid was also employed in several series of experiments. This method, which was originally developed for hippuric acid, also determines any other ether-soluble nitrogenous compounds (*e.g.* phenaceturic acid) not decomposed by alkaline hypobromite. Both phenylacetic and benzoic acids are determined if conjugated with glycine while neither free benzoic, phenylacetic acid, nor the products of their conjugation with non-nitrogenous substances (*e.g.* glycuronic acid) are determined by this procedure. Since we were concerned not only with hippuric acid but also with phenaceturic acid, which is less readily soluble in ether than hippuric acid, the ether extraction was carried out for 2 hours, in order to be certain that all of the phenaceturic acid was extracted.

In the last series of experiments, the Kingsbury-Swanson modification (6) of the Folin-Flanders method for total benzoic acid (free and combined) was used in addition to the other methods. *Total phenylacetic acid* is obviously included in this determination since it and its conjugated derivatives react similarly to benzoic acid. The oxidizing agents employed will also convert some phenylpyruvic acid if present to phenylacetic acid which will be extracted and titrated. Any other chloroform-soluble acids present in the hydrolysate will be determined also.

Because of the failure of the Wayne sublimation method to

¹ The phenyllactic acid used in these control experiments was prepared by Dr. Julius White. A grant from the Faculty Research Fund made possible the preparation of this substance.

estimate with sufficient accuracy small amounts of benzoic and phenylacetic acids, a modification of the method of Virtanen and Pulkki (7) for the estimation of volatile acids was developed. This method depends upon the principle utilized in the well known Duclaux method for the determination of the volatile fatty acids, that a *definite fraction* of a volatile substance is distilled from an aqueous solution in a *given time* under *specified conditions*. Variations in concentration within wide limits and the presence of other volatile substances do not affect this constant rate of distillation. In this method 200 cc. of the solution to be analyzed are placed in a 300 cc. Erlenmeyer flask fitted to a condenser and the rate of boiling so adjusted that 100 cc. of distillate are collected in 1 hour. The distillate and original solution are titrated with standard alkali. Under these conditions according to Virtanen and Pulkki, 4.8 per cent of the total phenylacetic acid and 17.2 per cent of the total benzoic acid present are found in the distillate. In our experiments in which the amounts of phenylacetic acid ranged from 60 to 100 mg., we obtained values ranging from 4.7 to 5.0 per cent, and with 40 to 125 mg. of benzoic acid, values from 16.9 and 17.3 per cent. With mixtures of these two acids, the amount of each may be calculated from a formula involving the titration values of the distillate and the original solution. In Table I are presented some representative results of analyses of known mixtures of the pure acids, which show that the method can be used with a fair degree of accuracy, even when small amounts of the acids only are present.

In order to apply this method to the analysis of urine, the benzoic and phenylacetic acids must first be separated from the other acids present. This is done quite efficiently by the sublimation method of Wayne. The alcoholic solution of the sublimate may then be titrated for the Wayne determination and the acids then extracted with ether from the acidified titration mixture. The ether extract is evaporated, the residue is dissolved in water, and the distillation is carried out as above described. It is possible in this way to determine the phenylacetic and benzoic acids by two methods with the use of the same sample of urine. A mixture of known amounts of benzoic and phenylacetic acids in solution was titrated and the mixture of the free acids recovered from this solution of their salts by ether extraction of the acidified

624 Phenylalanine and Phenylpyruvic Acid

solution was distilled according to the Virtanen-Pulkki procedure. Typical results are presented in Table II. In both Tables I and II, there is observed a tendency for the phenylacetic acid values to be slightly high and for those for benzoic acid to be slightly low.

TABLE I

Estimation of Benzoic and Phenylacetic Acids in Mixtures, by the Method of Virtanen and Pulkki

Mixture used		Titration of distillate	Titration of original solu- tion	Benzoic acid calculated	Phenylacetic acid calculated
Benzoic acid	Phenylacetic acid				
mg.	mg.	cc. 0.05 N alkali	cc. 0.05 N alkali	mg.	mg.
16	16	0.53	4.97	14.8	17.3
16	80	0.96	14.35	13.3	82.8
40	80	1.68	18.20	39.9	79.4
40	80	1.63	18.25	37.0	83.1
50	50	1.79	15.32	51.7	46.6
60	60	2.05	18.45	57.2	61.8
80	40	2.47	18.92	77.0	43.0
80	16	2.30	15.30	77.5	17.8
100	50	3.21	23.50	102.8	45.6
12	20	0.45	4.92	10.6	21.7

TABLE II

Recovery of Benzoic and Phenylacetic Acids from Solutions of Their Salts by the Modified Virtanen-Pulkki Procedure

Present		Recovered	
Benzoic acid	Phenylacetic acid	Benzoic acid	Phenylacetic acid
mg.	mg.	mg.	mg.
20	20	14.0	21.0
15	25	12.0	22.0
20	100	16.5	97.8
100	20	89.5	23.0

These errors, however, are not sufficiently great to affect the value of data obtained by this method in animal experiments, especially if the values are considered in relation to the values obtained by the other methods discussed.

For the identification of phenylpyruvic acid in the experimental

urines, the semicarbazone derivative was prepared. The urines, acidified and shaken with Lloyd's reagent to remove as much pigment as possible, were saturated with solid ammonium sulfate and extracted with ether until the ether extracts no longer gave a green color with dilute ferric chloride solution. The acid was then removed from the ether extracts by shaking with a small volume of dilute alkali. In this way it was possible to concentrate the phenylpyruvic acid from a considerable volume of urine. On acidification of the alkaline solution and addition of semicarbazide hydrochloride, the semicarbazone was obtained.

DISCUSSION

Six rabbits were used in this series in which seven experiments with *l*-phenylalanine,² eight with *dl*-phenylalanine, three with sodium phenylpyruvate, and one with phenylethylamine were carried out. In addition, as controls of the experimental procedure, benzoic acid was administered in two experiments and phenylacetic acid in three. Results typical of those obtained in all the experiments are presented in Tables III and IV. The figures presented for the normal control periods represent the averages of a large number of 48 hour periods since each experimental period was preceded and followed by at least one, usually two, control periods. Thus the control figures for Rabbits 3 (Table III) and 4 (Table IV) are averages of the values obtained in eighteen and twenty-one periods respectively.

Increases in the weight of the sublimate of the Wayne method were obtained after the oral or subcutaneous injection of *dl*-phenylalanine (Table III). The ingestion of *l*-phenylalanine, on the other hand, resulted in slight increases only in the weight of the sublimed acids. In three experiments with Rabbit 2 (only one of which is recorded in Table III) in which the natural optical isomer of phenylalanine was fed, the change in the weight of the sublimate was too small to be of significance. With

² The *l*-phenylalanine was presented to us by Dr. Seiichi Izume of the Central Laboratory of Dairen, Manchuria, to whom we wish to express our appreciation. This product, obtained from wheat gluten as a by-product in the preparation of the condiment, ajinomoto, contained a small amount (about 2 per cent) of tyrosine as determined colorimetrically by the modified Millon reaction of Folin and Ciocalteu.

626 Phenylalanine and Phenylpyruvic Acid

other animals (e.g., Rabbit 3 (Table III)), the increase after *l*-phenylalanine was more marked. By far the greatest increases in the amount of sublimate were noted after oral administration of the sodium salt of phenylpyruvic acid. Increases in phenac-

TABLE III
Influence of Phenylalanine and Phenylpyruvic Acid on Excretion of Phenylacetic Acid

All results are calculated as the amounts of phenylacetic acid excreted in 48 hour periods. Unless otherwise indicated, the acids were administered orally.

Substance administered	Griffith method	Wayne method		
		Hydrogen peroxide*	Weight of sublimate	Titration of sublimate
	gm.		gm.	gm.
Rabbit 2, weight 3.2 kilos				
Average control.....	0.161	+	0.151	0.183
<i>l</i> -Phenylalanine, 4.0 gm.....	0.149	+	0.170	0.165
		—	0.170	0.163
<i>dl</i> -Phenylalanine, 4.0 gm.....	0.347	+	0.129	0.140
		+	0.566	0.625
		—	0.296	0.318
Rabbit 3, weight 3.7 kilos				
Average control.....	0.178	+	0.159	0.167
<i>l</i> -Phenylalanine, 4.0 gm.....	0.219	—	0.147	0.158
		+	0.311	0.318
		—	0.288	0.293
<i>dl</i> -Phenylalanine, 4.0 gm.....	0.261	+	0.638	0.635
		—	0.298	0.280
“ 4.0 “ subcutaneously.	0.246	+	0.548	0.588
		—	0.337	0.321
Sodium phenylpyruvate, 5.0 gm.....	0.677	+	1.003	0.972
		—	0.867	0.805

* A plus sign indicates that hydrogen peroxide was used in the Wayne method and a negative sign that the peroxide was omitted.

eturic (or hippuric) acid excretion as measured by the Griffith method were usually obtained in the same periods in which the weight of sublimed acids showed increase. Calculations by the Wayne method indicated that these increases in sublimate were

due to the presence of phenylacetic acid, but further and more satisfactory evidence of this is afforded by the data obtained by the

TABLE IV

Excretion of Phenylacetic and Benzoic Acids after Oral Administration of Benzoic and Phenylacetic Acids and of Phenylalanine and Phenylpyruvic Acid

Rabbit 4, weight 3.2 kilos. All results are expressed in terms of gm. of phenylacetic acid excreted in 48 hour periods unless otherwise indicated.

Substance fed	Griffith method	Kingsbury-Swanson method	Wayne method. Sublimate			
			Hydrogen peroxide*	Titration of sublimate	Virtanen distillation of acids in sublimate	
					Phenylacetic acid	Benzoic acid
	gm.	gm.		gm.	gm.	gm.
Average control period.....	0.191	0.242	+	0.181	0.068	0.058
			—	0.165	0.068	0.057
Benzoic acid, 0.400 gm.....	0.527†	0.601†	+	0.458†	0.086	0.311
			—	0.400	0.082	0.301
Phenylacetic acid, 0.440 gm.....	0.523	0.562	+	0.531	0.352	0.100
			—	0.493	0.335	0.089
“ “ 0.440 “	0.542	0.605	+	0.557	0.400	0.071
			—	0.567	0.416	0.076
<i>l</i> -Phenylalanine, 4.0 gm.....	0.352	0.444	+	0.392	0.211	0.059
			—	0.366	0.219	0.056
“ 4.0 “	0.266	0.395	+	0.348		
			—	0.265	0.137	0.089
<i>dl</i> -Phenylalanine, 4.0 gm.....	0.224	0.771	+	0.669	0.435	0.089
			—	0.372	0.242	0.063
“ 4.0 “	0.234	0.711	+	0.551	0.405	0.097
			—	0.296	0.162	0.082
Sodium phenylpyruvate, 5.0 gm.....	1.015	1.364	+	1.370	1.205	0.051
			—	1.232	0.977	0.039

* A plus sign indicates that hydrogen peroxide was used in the Wayne procedure; a negative sign, that hydrogen peroxide was omitted.

† Calculated as benzoic acid.

use of the modification of the Virtanen and Pulkki method previously described (Table IV).

In order to assure ourselves further as to the reliability of the results obtained by the Virtanen distillation procedure, control experiments in which benzoic and phenylacetic acids were fed were carried out (Table IV). In each case, the results were those anticipated; *i.e.*, a marked increase in amount of the benzoic acid content of the sublimate when benzoic acid was ingested, and a similar increase in phenylacetic acid after the administration of phenylacetic acid. We believe that the results of these animal experiments together with the control experiments on the accuracy of the method as applied to known mixtures of these acids already described indicate that the method may be depended upon to give significant results.

In the experimental periods in which phenylpyruvic acid was fed, the results obtained with the Virtanen procedure showed that the increases in the amount of the sublimate were almost entirely accounted for by the increase in the phenylacetic acid fraction. Since by this method, no significant increases in the benzoic acid of the sublimate could be demonstrated, the increased values in the Griffith method would appear to have been due to the excretion of phenaceturic acid rather than hippuric. The increase following the ingestion of phenylpyruvic acid was especially marked, the phenaceturic acid (calculated as phenylacetic acid) excretion being 1.015 gm. in a 48 hour period (Table IV, calculated from determinations by the method of Griffith). Confirmation of these results was obtained by the isolation of phenaceturic acid from the urine of some experimental periods. Thus in one experiment, after 2.5 gm. of sodium phenylpyruvate had been fed for 2 successive days, it was possible to isolate from the urine 0.4 gm. of phenaceturic acid which melted at 142° . From the isolated phenaceturic acid, phenylacetic acid with a melting point of 75.5° was obtained after hydrolysis. The evidence indicates that under our experimental conditions phenylpyruvic acid is converted in part to phenylacetic acid which is conjugated with glycine and excreted as phenaceturic acid in the urine. The evidence of the formation of phenaceturic acid after the administration of phenylalanine is not so convincing. In five experiments in which *dl*-phenylalanine was administered and the excretion followed by the Griffith method, an increase in phenaceturic acid by this method was observed in three instances (Table III) while

in two experiments (Table IV), the values were only slightly higher than those of the control periods. With *l*-phenylalanine the results in one experiment (Table IV) clearly indicated an excretion of phenaceturic acid, with slightly increased excretion in two others, and no evidence of any change in a fourth (Table III). It should be noted that one of these positive results was observed after subcutaneous injection.

After the ingestion of phenylalanine or phenylpyruvic acid, increases in the values obtained by the Wayne method (either in the weight of the sublimate or in the amounts calculated from the titration of the acidity of the sublimate) were always greater than the increases of phenylacetic as phenaceturic acid (Griffith method) for the same experimental period. It was apparent that some other substance also gave rise to phenylacetic acid in the Wayne procedure. An excretion of phenylacetic acid either free or in conjugation with a non-nitrogenous substance (*e.g.* glucuronic acid) would explain this difference. However, the conjugation of phenylacetic acid with glycine would seem to be nearly quantitative in our experimental animals since in neither of the two experiments reported in which phenylacetic acid was fed (Table IV) was there observed a greater increase in the amount of total sublimable organic acid (Wayne method) than in the phenylacetic acid as phenaceturic acid (Griffith method).

It seems probable, therefore, that the presence in the urine of phenylpyruvic acid, which had previously been shown to be converted in large part to phenylacetic acid in the oxidation incidental to the Wayne method, was responsible for the different values obtained by the Wayne and Griffith methods. One of us (8) has presented evidence obtained by colorimetric methods of the excretion of significant amounts of this substance after the administration of phenylalanine. In the present series of experiments, we have been able to prepare the semicarbazone and hydrazone of phenylpyruvic acid from a number of urines secreted during experimental periods in which *dl*-phenylalanine, *l*-phenylalanine, and sodium phenylpyruvate were fed. Less direct evidence was depended upon to prove the presence of the same keto acid in other experimental urines. This evidence was supplied partly by the ferric chloride test which was positive for the urines of the experiments in which the keto acid was isolated as well as for the

urines of all other experiments in which phenylalanine or phenylpyruvic acid was fed or injected. In many experiments in which a strongly positive ferric chloride test was given by the urine, an increased value in the creatinine determination was also observed. The apparent increase of this urinary constituent was due, no doubt, to the presence of phenylpyruvic acid, which previous workers in this laboratory (8) found to be a source of color production in this colorimetric determination. There is little doubt then, that phenylpyruvic acid, present in the urines of experimental periods showing increased sublimates, was the chief source of the phenylacetic acid determined in the Wayne method and not arising from phenaceturic acid.

Approximate estimates of the amounts of the keto acid excreted may be made from the data. Possible sources of the increase of phenylacetic acid in the sublimate of an experimental period were phenylpyruvic acid, phenyllactic acid, and phenaceturic acid. The increase of phenylacetic acid derived from phenaceturic acid (Griffith method) may be subtracted from the total increase of phenylacetic acid, to give as the difference the extra phenylacetic acid derived from phenylpyruvic acids. Estimations by this method show that the largest amounts of the keto acid were excreted after the ingestion of the acid itself, the next largest after *dl*-phenylalanine, and the least after *l*-phenylalanine. The intensities of the green color in the ferric chloride test were in agreement with those estimations. Attempts to eliminate the influence of the keto acid on the sublimate were made by omitting the use of the oxidizing reagent in the procedure. The results of these experiments were of little value from the quantitative standpoint since appreciable amounts of phenylacetic acid were derived from phenylpyruvic acid even under these conditions. However, the difference in the results obtained with and without the use of the oxidizing agent (Tables III and IV) demonstrates that a substance yielding phenylacetic acid on oxidation was present in considerable amounts and thus offers indirect evidence of the presence of phenylpyruvic acid³ confirmatory of the other evidence of the presence of this catabolite.

³ It is of course possible that some of this phenylacetic acid was derived not from phenylpyruvic acid, but from phenyllactic acid formed in intermediary metabolism. In view of the relatively smaller amounts of sub-

In one experiment (Table III) *dl*-phenylalanine was injected subcutaneously. The result obtained was in all respects very similar to those obtained in the corresponding feeding experiments. This result shows that the changes observed in the feeding experiments were not due to microorganisms of the intestine. In one experiment not shown in the tables (*cf.* also Table V), sodium phenylpyruvate was injected subcutaneously and the urine analy-

TABLE V

Effect of Oral Administration of Phenylethylamine Hydrochloride on Excretion of Phenylacetic and Benzoic Acids

Rabbit 6, weight 3.0 kilos. Data are expressed as gm. of phenylacetic acid excreted in 48 hour periods, except where otherwise indicated.

Period	Griffith method	Wayne method. Titration of sublimate	Virtanen method	
			Phenylacetic acid	Benzoic acid
	gm.	gm.	gm.	gm.
Control.....	0.234	0.162	0.072	0.050
“.....	0.213	0.147	0.077	0.047
Experimental*.....	1.166	1.430	1.370	0.022
Control.....	0.904	1.147	1.083	0.020
“.....	0.136	0.147	0.117	0.021
“.....	0.168	0.149	0.082	0.046
Experimental†.....	0.243	0.601	0.509	0.051
Control.....	0.123	0.111	0.060	0.028
“.....	0.135	0.149	0.090	0.033

* 1.9 gm. of phenylethylamine hydrochloride were fed daily on 2 successive days.

† 2.5 gm. of sodium phenylpyruvate were injected subcutaneously on 2 successive days.

zed by the Griffith method. The phenaceturic acid excreted in a preceding control period was 0.301 gm., while after the injection it was 0.488 gm. This increase is much less than those which

limate obtained from phenyllactic acid in check experiments with the Wayne method as already described *particularly in the absence of peroxide*, we believe that little of the phenylacetic acid was derived from this source. Further evidence in support of this belief that phenylpyruvic acid is the more important intermediary catabolite is the parallelism between the intensity of the ferric chloride reaction and the amount of sublimate. Phenyllactic acid gives only a faint yellow-green color with ferric chloride.

occurred in the two experiments in which the same amount of phenylpyruvic acid was given orally, and suggests a formation of phenylacetic acid in the gastrointestinal canal from phenylpyruvic acid in the latter experiments. Some formation of phenaceturic acid appears to have occurred after injection of the keto acid, however. More complete data from other injection experiments will be necessary to permit an explanation of this difference.

In view of the possibility of decarboxylation, suggested by Thomas (9) as a possible intermediary step in amino acid catabolism, by which phenylethylamine would be derived from the phenylalanine, a study of the behavior of this amine in metabolism was made. Guggenheim and Löffler (10) have demonstrated the oxidation of this substance to phenylacetic acid in the body but no quantitative study appears to have been reported. Phenylethylamine hydrochloride was fed to a rabbit and analyses similar to those already discussed were carried out. The results (Table V) showed a marked increase in the excretion of phenylacetic acid by all the analytical methods used. This increased excretion as measured by the Wayne sublimation and Virtanen distillation methods was approximately the same, 2.28 and 2.30 gm. respectively. This corresponds to a recovery of approximately 78 per cent of the phenylacetic acid which could be derived from the oxidation of the amine to phenylacetic acid. By far the greatest part of the "extra" phenylacetic acid was eliminated as an ether-soluble nitrogenous compound (*i.e.*, phenaceturic acid) as shown by the analyses in which the Griffith method was used. 1.76 gm. of "extra" phenylacetic acid as determined by this method were excreted corresponding to about 60 per cent of the amount of phenylacetic acid which could be derived by oxidation of the amine fed. That the increased excretion as shown by the Virtanen method was due to the presence of phenylacetic was proved by the isolation of 0.4 gm. of phenylacetic acid with a melting point of 76° from a part of the solution distilled. No unchanged phenylethylamine could be detected in the urines. When solutions of the amine were subjected to the procedures of the Wayne and Griffith methods, no appreciable amounts of the substances determined by these methods were formed. In view of these results, phenylethylamine would appear to be readily converted to phenylacetic acid or its derivatives in the animal body.

Therefore, if, as suggested by Thomas (9), phenylalanine undergoes decarboxylation prior to deamination, complete oxidation of the amino acid would not occur.

One result of these experiments has been the demonstration of the conversion of phenylalanine to phenylpyruvic acid and the excretion of the latter in the urine, a result in agreement with previous data obtained by colorimetric methods in this laboratory (8) and with the work of Kotake and his coworkers (11). The most reasonable explanation of this failure of complete oxidation of the benzene ring is that, as a result of the deamination of phenylalanine, the keto acid is formed so rapidly that the disruption of the benzene nucleus cannot be effected and the intermediate product is excreted. A criticism may be raised in both series of experiments that the amounts of amino acids ingested were beyond the amounts which would be present in the organism under normal conditions of metabolism. Such a criticism may be warranted as far as concerns the experiments of the Japanese investigators, in which 9 gm. of phenylalanine were administered daily to rabbits of about 2.5 kilos of body weight and the administrations were continued for several successive days in some experiments. In our own experiments, however, considerably less than 1 gm. of phenylalanine per kilo of body weight was fed for 2 successive days only. In a further attempt to answer this possible criticism, a series of experiments was undertaken to determine the minimal amount of phenylalanine, which when fed to a rabbit of about 3 kilos of body weight would result in the excretion of phenylpyruvic acid in such amounts that it could be detected by the ferric chloride color reaction. A strongly positive test was obtained from urine collected 1 hour after the administration of 0.150 gm. of *dl*-phenylalanine, but no test could be observed after administration of smaller amounts of the amino acid. The ingestion of much larger amounts of *l*-phenylalanine was required in order to obtain a positive ferric chloride test in the urine. In similar experiments, phenylpyruvic acid could be readily detected colorimetrically in the urine 1 hour after feeding 0.160 gm. of phenylpyruvic acid as the sodium salt.

In our experiments much of the α -keto derivative formed from phenylalanine was excreted without further oxidation, but a small portion appears to have been converted to phenylacetic acid and excreted as phenaceturic acid. Although the formation of phenyl-

acetic acid in intermediary metabolism does not appear to have been demonstrated previously for phenylalanine, similar reactions which involved amino acids foreign to the body have been reported (12, 13). It is possible that the presence of amounts of phenylalanine, greater than those which might be expected to be formed in the course of the normal metabolism, may have resulted in a series of intermediary reactions, distinct from the normal, and that in our experiments, we were concerned essentially with an amino acid foreign to the organism. The detection of increased amounts of the acetic acid derivative after the administration of small amounts of phenylalanine would aid materially in determining whether phenylacetic acid may originate from phenylalanine in normal metabolism, but unfortunately, adequate methods for the determination of small amounts of phenylacetic acid or its conjugated derivatives are not available. It may be pointed out, however, that phenaceturic acid has been isolated as a constituent of normal urine of many species (14, 15). Our analyses of the sublimable organic acids of the control periods by the modified Virtanen procedure (Table IV) have indicated that these organic acids may consist of phenylacetic and benzoic acids in nearly equal amounts. Since the solubilities of hippuric and phenaceturic acids in organic solvents are very similar, it seems probable that of the material in the normal urine determined and calculated as hippuric acid, a considerable part may be phenaceturic acid.

Finally it should be pointed out that these experiments offer no evidence, positive or negative, concerning the formation of phenyllactic acid as an intermediary catabolite in the metabolism of phenylalanine. Certain observers (16) have isolated phenyllactic acid in the urine after the administration of phenylpyruvic acid and have thus demonstrated a reversal of the normal oxidative process. Our results have shown that in rabbits phenylpyruvic acid may be excreted in significant amounts after the administration of both phenylalanine and phenylpyruvic acid and that under the conditions of our experiments some of this phenylpyruvic acid may be oxidized to phenylacetic acid. Our experiments emphasize anew that if ready and complete oxidation of the benzene ring in phenylalanine is to be effected, the initial disruption of the ring must occur before the side chain is oxidized.

SUMMARY

1. A study has been made of the application of the method of Virtanen and Pulkki (7) to the quantitative determination of phenylacetic and benzoic acids or their conjugated derivatives in the urine in connection with the intermediary metabolism of phenylalanine.

2. On a diet of cabbage and oats, rabbits excreted approximately equal amounts of phenaceturic and hippuric acids as determined by the method of Virtanen and Pulkki.

3. Evidence of the presence of considerable amounts of phenylpyruvic acid in the urine of rabbits after oral administration or subcutaneous injection of 2.0 gm. of phenylalanine or an equivalent amount of phenylpyruvic acid was obtained. More of this keto acid was excreted after the administration of *dl*-phenylalanine than after the administration of *l*-phenylalanine.

4. A slight increase in the excretion of phenaceturic acid was observed in some experiments after oral administration or subcutaneous injection of 2.0 gm. of phenylalanine and a greater increase after the administration of an equivalent amount of phenylpyruvic acid. Under the same conditions no significant increase of hippuric acid excretion was observed.

5. These results would indicate that in the case of phenylalanine, α oxidation may follow oxidative deamination to give products which are not oxidized further in the organism. It is usually assumed that following oxidative deamination of phenylalanine, opening of the benzene ring occurs before further chemical changes in the side chain. This is apparently not the only path of catabolism open. The results of the present study indicate that the benzene ring of phenylalanine is oxidized less readily than has usually been assumed.

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636 Phenylalanine and Phenylpyruvic Acid

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